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The Structure of Bactoprenol, a Lipid Formed by Lactobacilli from Mevalonic Acid

BY KAREEN J. I. THORNE AND E. KODICEK
Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council

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1. The name 'bactoprenol' has been given to the most abundant lipid formed by three species of lactobacilli from mevalonic acid. 2. A method for the preparation of pure bactoprenol is described. 3. The thin-layer chromatographic properties of bactoprenol and of its acetylated and hydrogenated derivatives resembled those of dolichol. 4. Analysis by mass spectrometry and by nuclear magnetic resonance showed that the molecule is formed by condensation of 10 unsaturated isoprene units and 1 saturated isoprene unit. 5. Its molecular weight is 768 and it has 10 double bonds/molecule. 6. Infrared spectroscopy and the uptake of acetyl groups indicated that the molecule contains a hydroxyl group. 7. It is concluded that bactoprenol is a C_{55} isoprenoid alcohol.

Mevalonic acid, now known to be an intermediate in the biosynthesis of cholesterol, carotenoids and the isoprenoid side chains of ubiquinone, vitamin K and tocopherols, was originally discovered as a compound that would replace acetate in the growth of *Lactobacillus acidophilus* (Skeggs *et al.* 1956). *L. acidophilus*, *L. casei* and *L. arabinosus* have all been shown to utilize mevalonic acid for the synthesis of unsaponifiable lipids that differ from those mentioned above (Thorne & Kodicek, 1962*b*). The chemical structure of the most abundant of these lipids has now been elucidated and the name 'bactoprenol' is suggested for it. A preliminary account of some of the work described below has already been published (Thorne & Kodicek, 1963).

MATERIALS AND METHODS

Bacteriology. *L. casei* was used as the source of bactoprenol. Cells were grown for 48hr. at 37° in 24l. batches of a semi-synthetic medium (Thorne & Kodicek, 1962*a*,*b*).

Purification of bactoprenol. After being harvested, the washed wet cells were heated with 500ml. of 80% (v/v) ethanol until the volume was reduced to about 100ml. Water was added to give a total volume of 200ml. and the suspension was extracted three times with 200ml. of diethyl ether. Emulsions were broken by centrifuging. The ether extracts were dried by passing through anhydrous Na_2SO_4 on a sintered-glass funnel. The ether was removed *in vacuo*. The extracted lipid was dissolved in 5ml. of hot ethanol and left to stand overnight at room temperature. Precipitated lipid was discarded.

The supernatant was evaporated to dryness *in vacuo*, dissolved in a small volume of *n*-heptane and chromatographed on a column of alumina deactivated with acetic acid (Thorne & Kodicek, 1962*b*). The fraction eluted with 280ml. of 20% (v/v) benzene in *n*-heptane was collected and evaporated to dryness.

This fraction was acetylated with 0·4ml. of pyridine plus 0·2ml. of acetic anhydride at room temperature overnight. The excess of acetic anhydride and pyridine was removed in a stream of N_2. The acetylated material was chromatographed on a second solumn of alumina deactivated with acetic acid. The fraction eluted with the first 40ml. of *n*-heptane was discarded; the next 100ml. of *n*-heptane was collected and evaporated to dryness *in vacuo*. Heptane for this column was freed from ketones by distillation from 2,4-dinitrophenylhydrazine (4g. and 1ml. of conc. HCl/l.). The second heptane fraction contained the principal lipid formed by *L. casei* from mevalonic acid, as an acetylated derivative.

Thin-layer chromatography. Thin-layer chromatograms were prepared from Kieselgel G (E. Merck A.-G., Darmstadt, Germany) and developed with 10% (v/v) ethyl acetate in *n*-heptane.

Hydrogenation. Lipid samples were hydrogenated by the method of Bott, Eaborn, Peeling & Webster (1962). Samples in 0·1ml. of 95% (v/v) ethanol were added to 2·6mg. of tribenzylsilane and 0·5mg. of hexachloroplatinic acid in 2ml. of 95% (v/v) ethanol. The solution was kept at 30° and H_2 bubbled through for 90min., the ethanol level being constantly replenished. After hydrogenation the lipid was extracted three times with diethyl ether and the extracts were passed through Na_2SO_4 and evaporated to dryness *in vacuo*.

Molecular-weight determination by acetylation. Samples of partially purified bactoprenol obtained from the first alumina column were acetylated as described above by using [1-^{14}C]acetic anhydride of known specific activity. The acetylated lipid was chromatographed on the second alumina column and the eluted fraction was weighed and its radioactivity measured with a Tri-Carb scintillation counter

(Packard Instrument Co., La Grange, Ill., U.S.A.). From these results the weight of lipid reacting with one acetyl group could be calculated (Benson & Turner, 1960).

Methyl group determination. Free methyl groups were determined by oxidation of weighed samples of lipid to acetic acid with CrO_3–H_2SO_4 (Barthel & LaForge, 1944).

Iodine value. Iodine values were determined by Wijs's method (Jacobs, 1958).

Spectroscopy. Infrared spectra were measured with a Unicam SP.200 spectroscope. The mass spectrogram was obtained by Dr W. Kelly with A.E.I. equipment MS12, with direct-voltage scanning, and the sample was introduced by a direct scanning probe. Analysis by nuclear magnetic resonance was done by Mrs R. M. Lynden-Bell, with Perkin–Elmer equipment.

RESULTS

Purification. The procedure for the purification of the most abundant lipid formed by *L. casei* from mevalonic acid, bactoprenol, was devised with [2-^{14}C]mevalonic acid as marker. Examination of the total lipids formed from [2-^{14}C]mevalonic acid by reversed-phase paper chromatography had revealed the presence of at least four radioactive substances (Thorne & Kodicek, 1962*b*). The most abundant of these had R_F 0·15 in methanol–propan-1-ol–water (16:3:1, by vol.). Reversed-phase paper chromatography was therefore used to follow the purification of this material. The results of a sample purification are shown in Table 1. Each fraction was weighed and its radioactivity measured with a Panax thin end-window Geiger–Müller counter, the results being corrected for self-absorption. The final fraction represented 0·7% by weight of the total lipid and 28% of the radioactivity. Since analysis by reversed-phase paper chromatography had shown that this component contained 50% of the radioactive material this indicates that 44% of the lipid was lost during purification.

The purity of the final fraction was checked by reversed-phase paper chromatography and by thin-layer chromatography. After chromatographic separation only one radioactive spot could be detected and only one spot could be stained with iodine vapour or with 10% (v/v) ethanolic solution of phosphomolybdate in a 0·5–1·0mg. sample.

Thin-layer chromatography. Chromatography of bactoprenol on thin layers of Kieselgel G with 10% (v/v) ethyl acetate in *n*-heptane gave a spot of R_F 0·34. For comparison the R_F values of various other lipids in this solvent are as follows: progesterone, 0·13; phytol, 0·22; cholesterol, 0·26; dolichol, 0·34; vitamin K_2(20), 0·65; squalene, 0·96. Of these compounds only the C_{100} isoprenoid alcohol dolichol, isolated initially from animal tissues (Pennock, Hemming & Morton, 1960; Burgos, Hemming, Pennock & Morton, 1963), had the same R_F as bactoprenol. The colours given by bactoprenol and by dolichol after spraying the chromatograms with various reagents were compared (Table 2). Both substances gave positive reactions with iodine, rhodamine 6G and phosphomolybdate. The colours given with the other reagents were similar but not identical. Bactoprenol gave no reaction with triphenyltetrazolium chloride, bipyridyl–ferric chloride or alkaline *m*-dinitrobenzene.

The effects of aging, acetylation and hydrogenation on the chromatographic characteristics of ^{14}C-labelled bactoprenol were investigated. After treatment the radioactive lipid was chromatographed and radioactive spots were detected by radioautography. After leaving bactoprenol for 2 weeks at room temperature or for 3hr. in a stream of oxygen three additional slower radioactive spots could be detected, indicating that some breakdown had occurred. Acetylation gave a compound with R_F 0·73. The presence of acetyl groups in this compound was confirmed by using [1-^{14}C]acetic anhydride and unlabelled bactoprenol, when the same radioactive spot was observed. After hydrogenation all the radioactive material ran at the solvent front. Hydrogenation of radioactive dolichol gave the same results.

Molecular weight. The molecular weight of bactoprenol was measured by using [^{14}C]acetic anhydride. Each of five samples of impure bactoprenol, ranging in amount from 2·5 to 6·1mg., was acetylated with 0·02ml. of [^{14}C]acetic anhydride (specific activity

Table 1. *Purification of bactoprenol from a 24l. batch of* L. casei

Experimental details are given in the text.

	Yield (mg.)	Radioactivity (counts/min.)	Specific activity (counts/min./mg.)
Extracted lipid	105	7950	76
Ethanol-soluble lipid	75	7950	106
Fraction eluted from first alumina column	2·4	4000	1670
Fraction eluted from second alumina column	0·7	2250	3210

Table 2. *Colours given by bactoprenol and dolichol on spraying with various reagents after thin-layer chromatography*

Experimental details are given in the text.

	Bactoprenol		Dolichol	
	Daylight	Ultraviolet light	Daylight	Ultraviolet light
Iodine vapour	Brown	—	Brown	—
Rhodamine 6G	—	Yellow	—	Yellow
Phosphomolybdate*	Blue	—	Blue	—
Phosphoric acid*	Red	White	Rufous	White
$SbCl_3$	Rufous	Ochre	Rufous	Orange
$ZnCl_2$–benzoyl chloride*	Brown	White	Brown	Cream
Anisaldehyde–$SbCl_3$†	Mauve	Red	Purple	Orange

* Merck (1963).
† Katz (1960).

5740 counts/min./μmole) in the presence of 0·05ml. of pyridine. After acetylation and purification by chromatography on alumina the samples were weighed and one-tenth was counted with a Tri-Carb scintillation counter three times for 10min. The following results were obtained for the mass and radioactivity of the five acetylated samples: sample A, 0·49mg., 2180 counts/min., calculated mol.wt. 645; sample B, 2·23mg., 8320 counts/min., calculated mol.wt. 770; sample C, 1·18mg., 4020 counts/min., calculated mol.wt. 840; sample D, 0·56mg., 5370 counts/min., calculated mol.wt. 835; sample E, 1·07mg., 4880 counts/min., calculated mol.wt. 630. This gave a mean value for the molecular weight of 744 ± 45·3 for the acetylated compound, or 702 for the original bactoprenol. A more accurate measure of the molecular weight was obtained by mass spectrometry. The molecular weight as calculated from acetylation measurements is based on the assumption that bactoprenol contains only one acetylatable group/molecule.

Free methyl groups. The number of free methyl groups present in 2·1mg. of acetylated bactoprenol was determined by oxidizing them to acetic acid: 1m-mole of methyl group was found/72·1mg. of acetylated bactoprenol. This represents 11 methyl groups/molecule of acetylated bactoprenol. The accuracy of the method is such that this number is within experimental error of the expected number of 13 methyl groups/molecule in the acetylated substance. Oxidation of bactoprenol derived from [2-^{14}C]mevalonic acid gave acetic acid with no detectable radioactivity. As the volume of acetic acid produced was considerable, the minimum radioactivity detectable represented 10% of the total. The radioactivity of a single terminal methyl group derived from C-2 of mevalonic acid would not be detected at this level of sensitivity. It is deduced

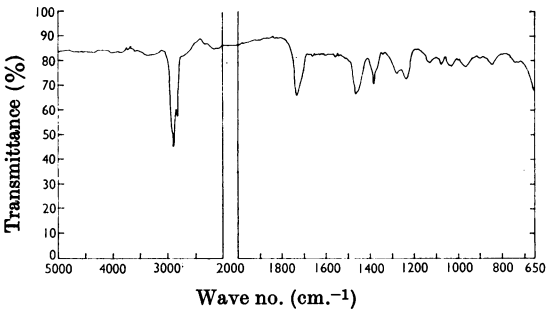


Fig. 1. Infrared spectrum of acetylated bactoprenol in a thin film. Experimental details are given in the text.

that the bulk of the methyl groups are not derived from C-2 of mevalonic acid. In addition, it was found that when the bacteria were grown in [*Me*-^{14}C]-methionine no radioactivity could be detected in bactoprenol, showing that methionine did not donate methyl groups for its synthesis.

Iodine value. The iodine value of bactoprenol was determined by Wijs's method: 17μequiv. of iodine was absorbed by a 0·66mg. sample of acetylated bactoprenol, or 2m-equiv./77·8mg.; this would correspond to 10·4 double bonds/molecule of mol.wt. 810, as determined by mass spectrometry.

Infrared spectrum. Fig. 1 shows the infrared spectrum of acetylated bactoprenol in a thin film. Peaks at 2840, 2920, 1460 and 1380cm.$^{-1}$ were attributed to CH_3 and CH_2 groups. Peaks at 1730 and 1230cm.$^{-1}$ could be explained by the acetyl group. Only a weak band at 840cm.$^{-1}$, given by trisubstituted olefins, was detected, and no absorption at 1680–1670cm.$^{-1}$. Possibly the high temperatures used for this work led to some destruction of

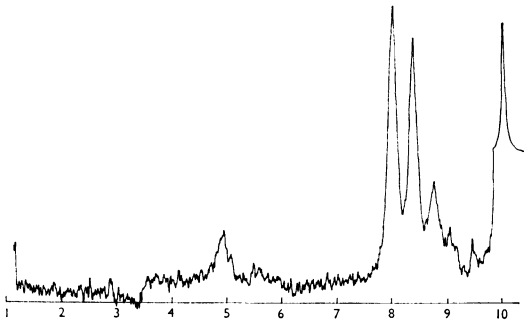


Fig. 2. Nuclear-magnetic-resonance spectrum of acetylated bactoprenol (60Mcyc./sec.). Experimental details are given in the text.

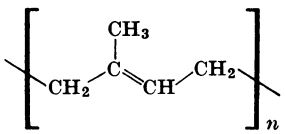
Table 3. *Mass spectrometry of acetylated bactoprenol showing the mass number of peaks obtained*

Experimental details are given in the text.

Mass number (*m/e*)			
810	367	259	107
750	355	245	95
707	341	231	93
681	327	217	81
613	315	203	69
545	313	189	68
477	304	175	67
409	299	167	55
398	289	161	43
395	281	149	41
381	273	135	29
370	267	121	27

the compound. Unexplained peaks occurred at 960, 1030, 1080, 1120 and 1280 cm.$^{-1}$. Bactoprenol exhibited little absorption in the ultraviolet region.

Nuclear magnetic resonance. A nuclear-magnetic-resonance spectrum, at 60Mcyc./sec., of acetylated bactoprenol (9·6mg. in 0·5ml. of chloroform) showed the following peaks on the τ scale (Fig. 2): 4·9p.p.m. for CH=C, 8·0p.p.m. for CH_2–C–C and 8·3p.p.m. for CH_3=C=C. The proportions of the areas under these peaks were 1:4:3, which could be explained by the isoprenoid structure (I). In addition, small peaks were found at 8·7, 9·0 and 9·4p.p.m.



(I)

The signal-to-noise ratio was too low for accurate determination of the relative sizes of these peaks. However, since the 8·7p.p.m. peak (C–CH_2–C and C⟩CH–C) was considerably greater than the 9·0p.p.m. peak (CH_3–C) the evidence favours the presence of a saturated isoprenoid group away from the ends of the molecule.

Mass spectrometry. In Table 3 are listed the peaks obtained when acetylated bactoprenol was examined by mass spectrometry. The peak at 810 represents the molecular weight of the acetylated lipid and 750 the molecular weight after removal of the acetyl group. These values are close to those obtained by acetylation and should be used as the accurate values for the molecular weights, which therefore amount to 768 for bactoprenol. A series of peaks 681, 613, 545, 477, 409, 341, 273, 203, 135 are characterized by successive removal of units of 68, the mass of an isoprene unit. In one case a mass unit of 70 is lost (from 273 to 203), which corresponds to a saturated isoprene unit. This is in agreement with the evidence for a saturated group obtained by nuclear magnetic resonance. Similar series differing from one another by 14 mass numbers and descending in units of 68 are 395, 327, 259, 189, 121 and 381, 313, 245, 175, 107 and 367, 299, 231, 161, 93 also occur. In each series there is one removal of a unit of 70 instead of 68. The occurrence of peaks at 43 and 41 suggests that the molecule has an isopropyl terminal group. The evidence presented here is in agreement with bactoprenol's having an isoprenoid structure formed by condensation of 10 unsaturated isoprene units and 1 saturated isoprene unit, with an isopropyl group at one end, and containing an acetylatable hydroxyl group.

DISCUSSION

Two-thirds of the mevalonic acid incorporated by lactobacilli is found in the unsaponifiable lipid fraction. Half of this is located in a single compound. The evidence presented here suggests that this substance, bactoprenol, is a branched-chain compound formed by the condensation of 10 unsaturated isoprene units and one saturated isoprene unit, and contains a hydroxyl group. The structure of the hydrocarbon chain is confirmed by its nuclear-magnetic characteristics and by mass spectrometry, which indicate a molecule of 11 isoprene units, one saturated, with an isopropyl group and an acetylatable hydroxyl group.

The presence of a hydroxyl group is suggested by the ability of the molecule to take up an acetyl group. As no nitrogen could be detected it is assumed that the acetylatable group is hydroxyl. No other polar groups were apparent in its infrared

spectrum. The analysis of the infrared spectrum indicated trisubstituted olefins, but only weakly, and CH_3 and CH_2 groups. Since the free alcohol could not be recovered from the ester in sufficient quantity no information could be obtained about the hydroxyl group. A molecular weight of 768 for bactoprenol determined by mass spectrometry is in good agreement with the molecular weight obtained from the mass of lipid reacting with one acetyl group. It is deduced from this that the molecule contains only one hydroxyl group. This molecular weight is lower than that previously reported (Thorne & Kodicek, 1963) owing to further purification of the lipid.

Bactoprenol has a structure similar to the isoprenoid alcohol dolichol (Pennock *et al.* 1960), which has been found in liver, kidney, pancreas, spleen, brain, intestine and skeletal muscle (Burgos *et al.* 1963) and in the spadix of *Arum maculatum*. It resembles dolichol in R_F and staining characteristics on thin-layer chromatography and differs from it in size. A C_{110} isoprenoid alcohol was isolated from *Aspergillus fumigatus* (Burgos, Butterworth, Hemming & Morton, 1964); the synthesis of geranylgeraniol by extracts of *Micrococcus lysodeikticus* has been described (Kandutsch, Paulus, Levin & Bloch, 1964). As yet little is known about the functions of any of these isoprenoid alcohols in their widely different organisms, but it is possible that the formation of bactoprenol, the most abundant of the four metabolites of mevalonic acid, is necessary for growth of lactobacilli. It appears that it is incorporated into a lipoprotein (Thorne & Kodicek, 1962*a*,*b*,*c*).

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